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Microsporidia Infect the *Liophloeus lentus* (Insecta, Coleoptera) Ovarioles, Developing Oocytes and Eggs

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In the ovarioles of *Liophloeus lentus* (Insecta, Coleoptera, Curculionidae) two types of bacteria and parasitic microorganisms belonging to Microsporidia have been found. This study shows that the different microsporidian life stages (meronts, sporonts, sporoblasts and spores) infect the outer ovariole sheath, trophic chambers, follicular cells, late previtellogenic and vitellogenic oocytes and eggs. In trophic chambers the parasites are very abundant and are distributed unevenly, i.e. their large mass occupies the syncytial cytoplasm between the nurse cell nuclei, whereas the neck region of the trophic chamber (which houses young oocytes, prefollicular cells and trophic cords) is almost free of parasites. The developing oocytes and eggs contain a lower number of parasites which are usually distributed in the cortical ooplasm. The gross morphology of the ovaries is similar in infected and non-infected specimens. Similarly, the presence of a parasite seems to not disturb the course of oogenesis. The only difference was found in the ultrastructure of mitochondria in young previtellogenic oocytes. In the infected females they are unusual i.e. bigger and spherical with tubular cristae, whereas in the non-infected insects they are elongated and have lamellar cristae. As oogenesis progresses the unusual mitochondria rapidly change their morphology and become similar to the mitochondria in non-infected females. Taking into account the distribution of parasites within the ovarioles, it is suggested that they infect growing oocytes via outer ovariole sheath and follicular epithelium rather than via trophic cords.

Key words: Microspora, transovarial transmission, insects, parasites, oogenesis, ovary.

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Numerous prokaryotic and eukaryotic microorganisms infect many arthropod species. Some are obligate intracellular agents, both symbiotic and parasitic, which can not live outside the host cells. Endosymbiotic microorganisms are characteristic e.g., for insects with restricted diet and they are usually transmitted transovarially from one generation to the next (BUCHNER 1965; DOUGLAS 1989; WERREN & O'NEILL 1997). Arthropods are also infected by numerous intracellular parasites such as viruses, bacteria and protists and there is a growing number of papers presenting data on their vertical transmission (e.g., KELLEN *et al.* 1966; ANDREADIS & HALL 1979; GINSBURGER-VOGEL & DESPORTES 1979; BECNEL *et al.* 1989; DICKSON & BARR 1990; VALE *et al.* 1992; DUNN *et al.* 1995; NI *et al.* 1997; TERRY *et al.* 1997, 1999, 2004; DUNN *et al.* 2001).

Vertically transmitted parasites pass from host generation to offspring; therefore the transmission takes place within the host lineage. In mammals

the parasites invade offspring via the placenta (e.g. HIV virus, *Plasmodium*, *Toxocara canis* – REDD *et al.* 1996; SAGLIO *et al.* 1996; KASSAI 1995), whereas in egg laying animals the parasites pass from mother to embryo via egg cytoplasm or on the surface of egg envelopes (e.g. Ross River virus, haplosporidian-like parasite and many microsporidian species – VALE *et al.* 1992; GINSBURGER-VOGEL & DESPORTES 1979; FINE 1984; ANDREALIS 1987; SMITH & DUNN 1991; DUNN *et al.* 1995). The vertical transmission is uniparental (maternal) and it can be compared to maternal inheritance of cell organelles, such as mitochondria.

It has been demonstrated that in insects a wide range of microorganisms such as viruses (VALE *et al.* 1992), bacteria (BUCHNER 1965; DOUGLAS 1989; WERREN & O'NEILL 1997), protists (GINSBURGER-VOGEL & DESPORTES 1979) and microsporidians (DUNN *et al.* 2001; TERRY *et al.* 2004) can pass transovarially from the mother to the off-

spring. The exact time and mode of oocyte (or egg) invasion by microorganisms are different and various strategies of invasion have been described (BUCHNER 1965; DOUGLAS 1989; DUNN *et al.* 2001). During studies on oogenesis in a weevil, *Liophloeus lentus*, two types of bacteria were found in ovarioles (at least one of which seems to be transovarially transmitted, ŚWIĄTEK *et al.*, in preparation) and a parasitic microorganism belonging to the phylum Microsporidia (Microspora). The Microsporidia is a very specialised group of intracellular parasites related to fungi (KEELING & MCFADDEN 1998; HIRT *et al.* 1999; KEELING & FAST 2002; KEELING 2003) found in many invertebrate (mainly in arthropods) and vertebrate (mainly in fishes) species (BIGLIARDI & SACCHI 2001; DUNN and SMITH 2001; KEELING & FAST 2002; DIDIER *et al.* 2004). Microsporidia have several life stages as meronts, sporonts, sporoblasts and spores. The life cycles of Microsporidia show a great diversity from direct (without intermediate host) to cycles with an intermediate host (see DUNN & SMITH 2001). Additionally, they can be transmitted only horizontally (many species), both horizontally and vertically and vertically exclusively (rare) (see DUNN *et al.* 2001; DUNN & SMITH 2001; DIDIER *et al.* 2004 for review).

In the present paper it is shown that microsporidian species that meet the criteria for the genus *Unikaryon* are present in different parts of ovarioles and in developing oocytes and eggs of *Liophloeus lentus*. Moreover, the mode of oocyte invasion by parasites is described and oogenesis is compared in infected and non-infected females.

Material and Methods

Adult specimens of *Liophloeus lentus* Germar, 1824 were collected from forest meadows in the Gorce mountains in southern Poland. The insects were collected in May 2004.

Light and electron microscopy

The ovaries were isolated and fixed in 2.5% glutaraldehyde in 0.1 M. phosphate buffer (pH 7.4) for several days. After washing in phosphate buffer, the material was postfixed for 1 hour in 1% OsO₄ in the same buffer, dehydrated in a graded series of ethanol and acetone, and embedded in Epon 812 (Fullam Inc., Latham, NY, U.S.A.). Semithin sections (0.7 µm thick) were stained with methylene blue and examined with an Olympus BX60 microscope. Ultrathin sections (70–80 nm) were cut on a Leica ultracut UCT ultramicrotome. After contrasting with uranyl acetate (10 min) and

lead citrate (15 min), the sections were examined in a Hitachi H500 electron microscope at 75 kV.

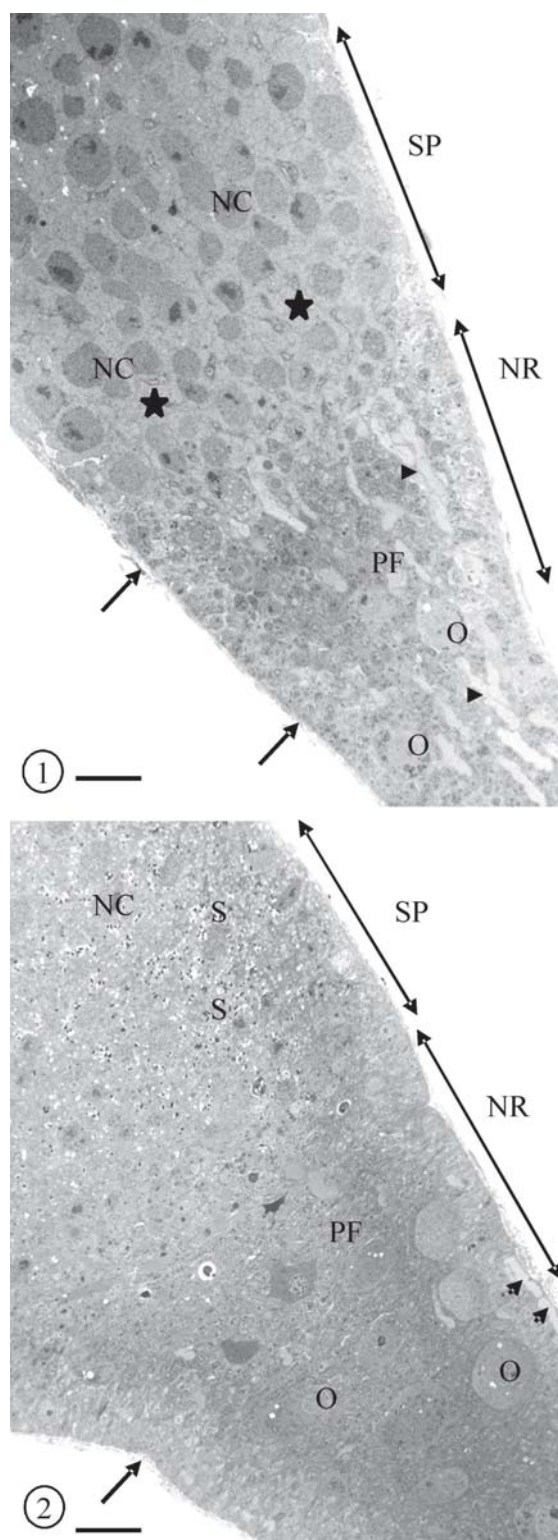
Results

Gross morphology of the ovary

The females of *L. lentus* have paired ovaries, each one comprises only two ovarioles. As in other previously described polyphagous beetles (see BÜNING 1979a, b; KING & BÜNING 1985; BÜNING 1994), the ovaries of the studied species are of the telotrophic meroistic type. The ovariole structure as well as the course of oogenesis in *L. lentus* are similar to those in other weevil species (*Phyllotribus urticae*, BIELAŃSKA-OSUCHOWSKA 1960; BÜNING 1979a; *Foucartia squamulata*, BILIŃSKI & PETRYSZAK 1978; *Anthonomus pomorum*, ŚWIĄTEK 1999, 2002) and their description is not the main aim of the present study. In a brief overview, each ovariole is composed of a short terminal filament (not shown), a large trophic chamber (tropharium) (Figs 1 & 2) and a vitellarium which is connected to a broad lateral oviduct (not shown). The ovariole is encompassed by a thin outer ovariole sheath (Figs 1 & 2, 10–13). The tropharium houses hundreds of nurse cell (trophocyte) nuclei embedded in a common cytoplasm (Figs 1–4), however, there are some remnants of cell membranes between trophocyte nuclei (Fig. 1). The somatic interstitial cells are scattered throughout the common cytoplasm. At the base of the trophic chamber the so-called neck region is present, containing young oocytes, prefollicular cells and profiles of the trophic cords which ensure cytoplasmic continuity between tropharium and developing oocytes (Figs 1 & 2). The vitellarium is occupied by 40–50 linearly arranged oocytes in consecutive stages of oogenesis, i.e. in previtellogenesis, vitellogenesis and chorionogenesis. Mature eggs are deposited in the broad lateral oviducts.

Microsporidia, their structure, distribution within the ovariole and their influence on oogenesis

The ovarioles of 2 of a total of 12 studied specimens of *L. lentus* were infected by microorganisms belonging to phylum Microsporidia. The tropharia of these infected specimens are loaded with a large number of different microsporidian life forms (i.e. meronts, sporonts, sporoblasts and spores) (Figs 2–4). The meronts occur rarely (not shown), sporonts are more abundant (Figs 3–5). Both cell types are usually roundish or slightly elongated cells encircled by a cell membrane (Figs 5 & 7). They are characterised by simple cellular



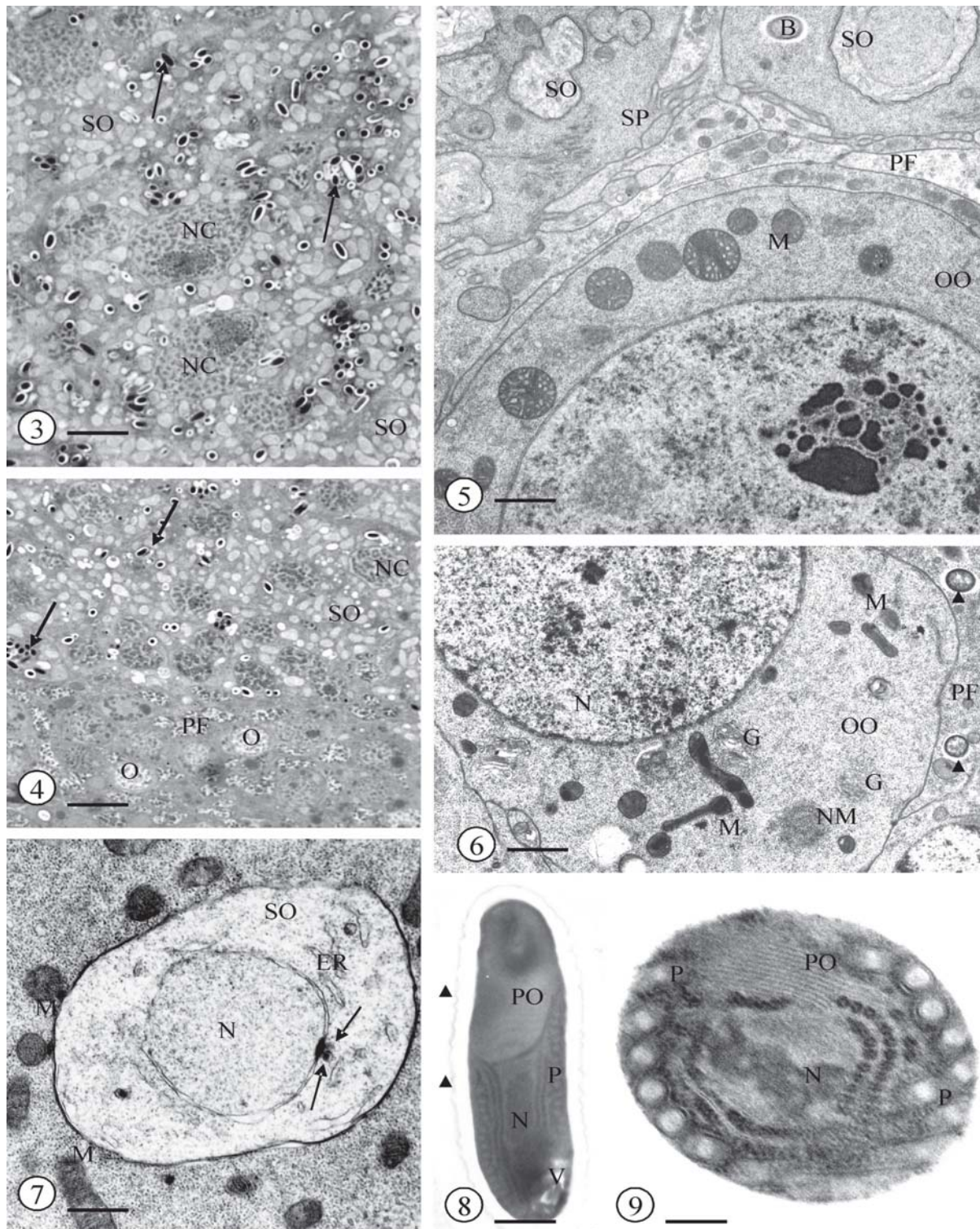
Figs 1-2. Fig. 1. Uninfected female. The syncytial part (SP) of the trophic chamber houses nurse cell nuclei (NC) embedded in a common cytoplasm (stars). In the neck region (NR) prefollicular cells (PF), young oocytes (O) and profiles of trophic cords (arrowheads) are visible. Arrows point to the outer ovariole sheath. LM, epon semithin section. Bar=140 μ m. Fig. 2. Infected female. In the syncytial part of the trophic chamber (SP) spores of *Unikaryon* sp. (S) are visible. The neck region (NR) is free of parasites. Arrow marks the outer ovariole sheath, arrowheads – trophic cords, NC – nurse cell nuclei, O – young oocytes, PF – prefollicular cells. LM, epon semithin section. Bar=106 μ m.

structure, i.e. they always have only one nucleus; in their cytoplasm endoplasmic reticulum and ribosomes can be observed (Fig. 7). No mitochondria or Golgi apparatus can be found. The sporoblasts are also uninucleate (not shown), they give rise to spores. The elongated spores are encompassed by a spore wall (Fig. 8). Furthermore, the spores contain elements characteristic for all Microsporidia such as an anchoring disc, polar filament, polaroplast, nucleus (always only one nucleus) and a posterior vacuole (Figs 8 & 9).

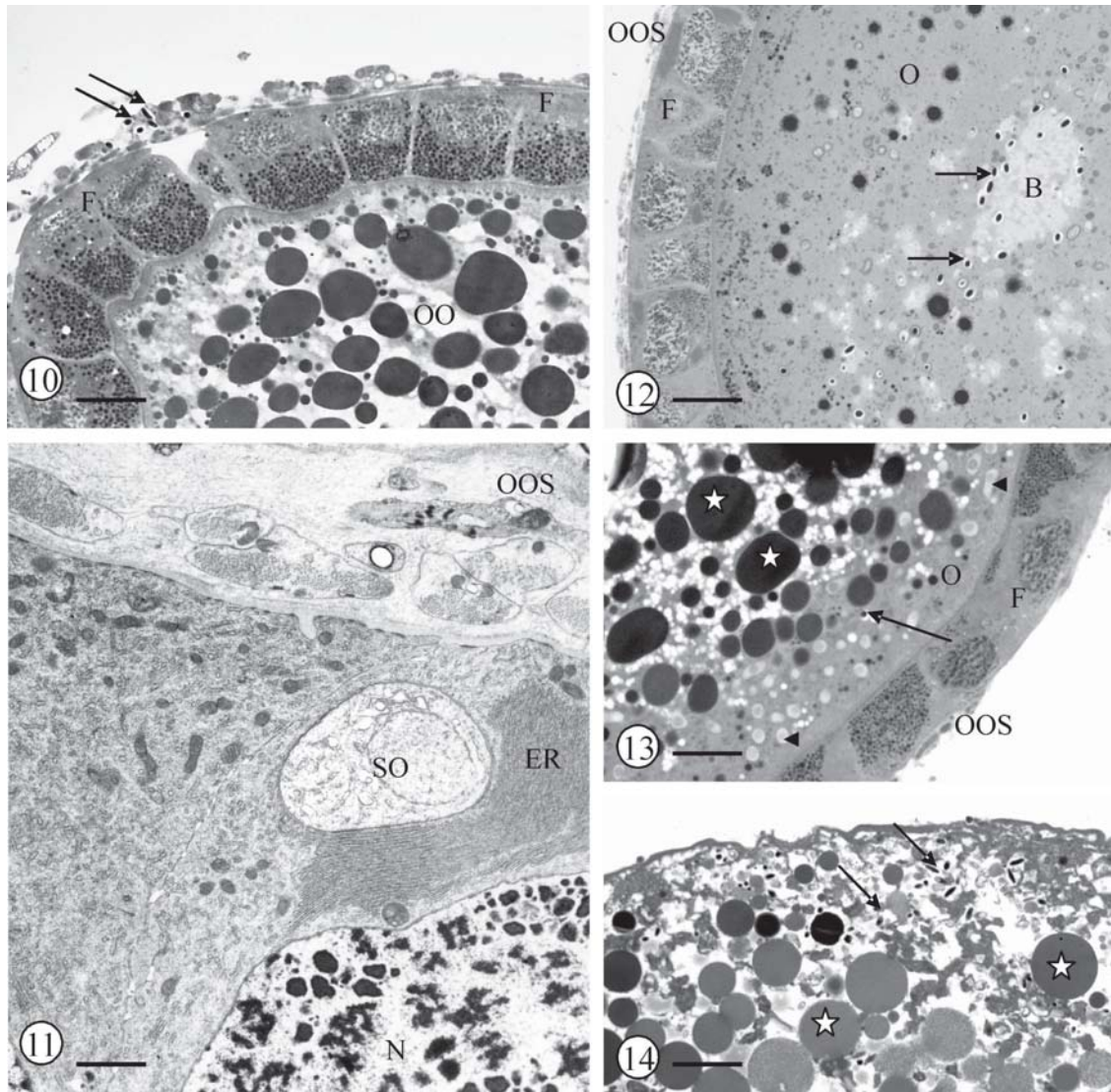
The distribution of the microsporidia within the tropharia seems to be uneven. Namely, all microsporidian life stages were found almost exclusively in the common cytoplasm, i.e. between the nurse cell nuclei (Figs 2-4), whereas only single spores were found inside the neck region (Fig. 2). Figures 2, 4 & 5 show the transition zone between the common cytoplasm filled with the microsporidia and the neck region which is almost free of parasites. Only single spores have been found in prefollicular cells, young oocytes and in trophic cords (not shown).

Within the vitellarium the different forms of parasite (sporonts, sporoblast and spores) were found inside the outer ovariole sheath (Fig. 10), in follicular cells (Fig. 11) and in late previtellogenic and vitellogenic oocytes (Figs. 12 & 13). Microorganisms were also found in egg cytoplasm (Fig. 14), however, it should be added that not all oocytes and eggs were infected with microsporidia. During late previtellogenesis/early vitellogenesis the sporonts and spores seem to be distributed randomly within the ooplasm, or they are associated with accumulations of endosymbiotic bacteria (Fig. 12). In late vitellogenic oocytes and in eggs they are distributed mainly in the cortical layer of ooplasm (Figs 13 & 14).

There are no detectable differences in the ovariole morphology and in the course of oogenesis between infected and non-infected specimens of *L. lentus* (Figs 1 & 2). The only differences were found in the ultrastructure of mitochondria in young previtellogenic oocytes laying in the neck region of the trophic chamber. Even though the young previtellogenic oocytes in the infected specimens are usually free from parasites, their mitochondria markedly differ from mitochondria in the same stage oocytes of the non-infected females. Namely, their mitochondria are much larger and oval in shape and their cristae are tubular (Fig. 5), whereas in young oocytes in the non-infected specimens they are smaller, oval or elongated and have lamellar cristae (Fig. 6). Interestingly, the mitochondria with tubular cristae change their morphology as oogenesis progresses and oocytes located in the anterior part of the vitellarium (i.e. just beneath the neck region) possess



Figs 3-9. Fig. 3. Infected female. The common cytoplasm in the trophic chamber is filled with a huge mass of sporonts (SO) and spores (arrows) of *Unikaryon* sp. NC – nuclei of nurse cells. LM, epon semithin section. Bar=17 μ m. Fig. 4. Infected female. The transition zone between the syncytial part of the trophic chamber filled with microsporidia (sporonts – SO and spores – arrows are visible) and the neck region in which young oocytes – O and prefollicular cells – PF are free from parasites. NC – nurse cell nuclei, LM, epon semithin section. Bar=47 μ m. Fig. 5. Infected female. Young previtellogenic oocyte located just beneath the syncytial part of the trophic chamber (SP). Note unusual mitochondria (M) in the ooplasm (OO). B – bacteria, PF – fragments of prefollicular cells, SO – sporonts. TEM. Bar=2.1 μ m. Fig. 6. Uninfected female. Young previtellogenic oocyte in the neck region. In the ooplasm (OO) elongated mitochondria (M), Golgi apparatus (G) and nuage material (NM) are visible. N – oocyte nucleus, PF – fragments of prefollicular cell with bacteria (arrowheads). TEM. Bar=1.4 μ m. Fig. 7. The sporont (SO) of *Unikaryon* sp. laying in the syncytial part of the trophic chamber. The sporont cell membrane is thick and is in close association with host mitochondria (M). The sporont has a single nucleus (N), the spindle plaque (arrows) is visible on the nuclear envelope. In the sporont cytoplasm free ribosomes and cisternae of endoplasmic reticulum (ER) are present. TEM. Bar=0.58 μ m. Fig. 8. The longitudinal section through the spore. The spore wall is hardly visible (arrowheads), N – nucleus, PO – polaroplast, P – polar filament, V – posterior vacuole. TEM. Bar=0.62 μ m. Fig. 9. The cross section through the spore just beneath the polaroplast (PO). Note the coils of polar filament (P) and nucleus (N). TEM. Bar=0.19 μ m.



Figs 10-14. Infected female. Fig. 10. A fragment of the vitellarium. Spores are visible within the outer ovariole sheath (arrows). F – follicular cells filled with precursors of egg envelopes, OO – ooplasm of the vitellogenic oocyte rich in yolk. LM, epon semithin section. Bar=59 μ m. Fig. 11. The sporont (SO) associated with host endoplasmic reticulum (ER) in the follicular cell. N – nucleus of the follicular cell, OOS – outer ovariole sheath. TEM. Bar=2.7 μ m. Figs 12, 13. The different life stages of *Unikaryon* sp. in the early vitellogenic (Fig. 12) and in the late vitellogenic oocyte (Fig. 13). Arrows – spores, arrowheads – sporonts, B – accumulation of symbiotic bacteria, F – follicular cells, O – ooplasm, OOS – outer ovariole sheath, white stars mark yolk spheres. LM, epon semithin section. Fig. 12 bar=50 μ m; Fig. 13 bar=48 μ m. Fig. 14. A fragment of a mature egg. Numerous spores (arrows) are visible. White stars – yolk spheres. LM, epon semithin section. Bar=16 μ m.

mitochondria similar to those in non-parasite infected specimens (not shown).

Discussion

Although horizontal transmission seems to be the main route of microsporidian transmission (CANNING & LOM 1986) there is a growing number of reports describing transovarial transmission of these parasites (see DUNN *et al.* 2001 for references; TERRY *et al.* 2004). These studies usually provide a detailed description of parasite developmental stages whereas much less is known about the influence of parasites on the course of oogenesis and how and when the oocytes are infected (e.g., BECNEL

et al. 1989; DICKINSON & BARR 1990). In addition authors rarely focus their attention to microsporidian invasion into oocytes and strategies ensuring normal oogenesis, much more is known about the various mechanisms that provide for safe transmission of parasites during embryogenesis (SAJAP & LEWIS 1988; KELLEN & LINDEGREN 1973; RAINA *et al.* 1995; TERRY *et al.* 1997, 1999; DUNN *et al.* 1998).

It is apparent that a transovarially transmitted parasite does not disturb such complex and sensitive processes as oogenesis and embryogenesis. This is also the case in the studied species. No differences were observed in ovariole morphology, appearance and number of oocytes growing in vitellaria and in the course of oogenesis in the infected and uninfected females of *L. lentus*. At

present, the observed difference in mitochondrial ultrastructure in the youngest oocytes can not be satisfactorily explained. It is known that microsporidia have no functional mitochondria but only their non-functional remnants (WILLIAMS *et al.* 2002), and there are many reports showing the close association between microsporidia and host mitochondria and endoplasmic reticulum (CALI & OWEN 1990; CANNING & HOLISTER; 1992; DE GRAAF *et al.* 1994; TERRY *et al.* 1997, 1999; this study). In the studied species unusual mitochondria with tubular cristae were found exclusively in the youngest oocytes, however, these oocytes were not infected by parasites! Additionally, as shown in Fig. 7, the mitochondria which interact with parasites (e.g. in trophic chamber) do not change their morphology at all. This phenomenon needs further studies.

A detailed analysis of the parasite life cycle (e.g., details of sporogony) is still in preparation, however, considering that all the studied microsporidian life stages are uninucleate (a diplokaryon has never been found) and that they possess typical spore wall structure, we preliminarily determined the studied microsporidian species to the genus *Unikaryon*. The microsporidia from the genus *Unikaryon* were at first described as hyperparasites of trematodes (CANNING *et al.* 1974). Later were found in the gut, Malpighian tubules, muscles, fat body and gonads of scotylids (KNELL & ALLEN 1978; WEISER *et al.* 1998, 2002) and chrysomelids (TOGUEBAYE & MARCHAND 1983, 1984). According to the authors knowledge, the transovarial transmission of microsporidians from the genus *Unikaryon* has not been described.

The distribution of *Unikaryon* sp. within *L. lentus* ovarioles and the route of oocyte invasion are of special interest. The most infected part of the ovariole is the main part of the trophic chamber which is occupied by the nurse cell syncytium. Despite the massive infection, this part of the tropharium seems to be unaffected, and looks similar to that of non-infected females and in other previously studied weevil species (BÜNING 1979a; ŚWIĄTEK 1999, 2002). In contrast, the neck region of the tropharium is almost free of parasites, only single spores occur in young oocytes, prefollicular cells and trophic cords. Again, the parasites (mainly spores) were observed in late previtellogenic and in older oocytes, however, a comparably extensive infection as that in tropharia was never observed in growing oocytes. This strategy of parasite distribution seems to be in accordance with the general rule that the parasite which is vertically transmitted must minimise its negative influence on host reproduction (DUNN *et al.* 2001).

In the present study, as well as in the other studied cases (DUNN *et al.* 2001), the direct invasion of oocytes by microsporidia was not observed. How-

ever, taking into account the distribution of parasites within the ovariole, it can be assumed that this microsporidian species infects oocytes via outer ovariole sheath and follicular cells rather than via trophic cords. As mentioned above, the young oocytes and trophic cords are very rarely infected, in contrast to many late previtellogenic and vitellogenic oocytes which contain sporonts and spores. Furthermore, all parasite life stages were found in the outer ovariole sheath and in the follicular cells. Because Microsporidia can not move actively, this infection route requires spore germination and sporulation in each passing cell (for a description of microsporidian life cycle see DUNN & SMITH 2001; KEELING & FAST 2002; DIDIER *et al.* 2004). Thus, the presence of the different life forms (i.e. sporonts, sporoblasts and spores) in ovariole sheath cells and in follicular cells supports this hypothesis. The infection of oocytes via the follicular cells seems to be a widespread mode of microsporidian invasion; however only in *Gammarus duebeni* has this process been described in detail (TERRY *et al.* 1999). On the other hand, we can not exclude that some spores can reach the growing oocytes via trophic cords or that the microsporidia infect the ovaries early in development as was also shown in *G. duebeni* (DUNN *et al.* 1995; TERRY *et al.* 1997, 1999), and are present at least in some germ-line cells even before oogenesis. To exclude this possibility, studies on *L. lentus* larvae are required.

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